Protein Complex Analysis Project (PCAP): High Throughput Identification and Structural Characterization of Multi-Protein Complexes During Stress Response in *Desulfovibrio vulgaris: Microbiology Subproject*

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VIMSS Virtual Institute for Microbial Stress and Survival — http://vimss.lbl.gov/

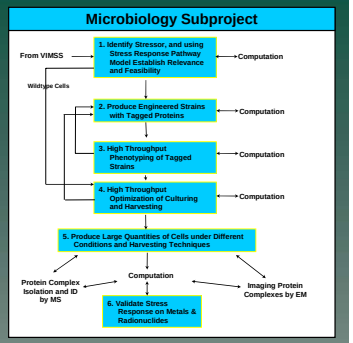
Mission

The Microbiology subproject is the source of environmental and microbiological expertise that determines the stress response pathways that will be studied, helps the computation group verify the stress response pathway models, develops engineered strains of *Desulfovibrio vulgaris*, establishes the phenotype and culture conditions most appropriate for the engineered strains, verifies stress response as it relates to metal and radionuclide reduction, and produces the large quantities of biomass needed for protein complex characterization and imaging by the other groups

Environmental Stress

The environment is the context in which genomes evolved, function, and continue to evolve. It is the only context in which they can be fully understood.

Microbiology Subproject



Stressors and Stress Response Pathways

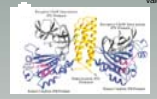
- DOE Relevant Environmental Stress Response Pathways in DvH studied by VIMSS to date: O_2, NO_3, **NO_2**, pH (5.5, 10), **NaCl, KCl**, Cr, **Heat** & Cold shock, **Growth Phase**
- Planned FY06
  - –DvH mutants repeat with previous stresses, DP4
  - –DvH stress with Cr, U reduction

Stress Response in Bacteria

In bacteria, two-component systems comprised of **Histidine Sensor Kinases** (HSKs) and **Response Regulators** (RRs), serve as the primary means for response to changes in the environment.

The *D. vulgaris* genome contains 64 ORFs annotated as HSKs.

To enable evaluation of stress response in *D. vulgaris*, a library HSK knock out mutants would be very valuable.

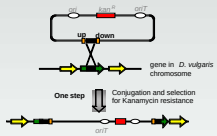


Producing Engineered Strains with Tagged Proteins

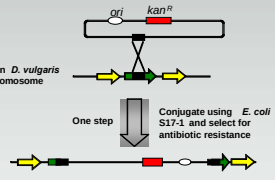
Genetics for Project
- **Tag proteins for complex isolation**
- **Tag proteins for imaging**
- **Create deletion strains**

Bar-coding

Incorporation of a set of unique sequences to tag the mutant



Single cross-over gene interruption



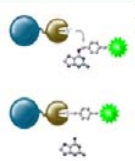
A vector for systematic gene inactivation in *Bacillus subtilis* . Vagner et al., Microbiology (1998), 144(11), 3097-3104.

Functional Characterization of the *S. cerevisiae* Genome by Gene Deletion and Parallel Analysis, (1999) Winzeler et al., SCIENCE VOL 285 6 AUGUST p 901

Tag Proteins for Imaging

The SNAP-Tag™ Technology

Reaction of SNAP-tag (gold) with para-substituted benzyl guanine, leading to covalent attachment of a label (green) to the protein of interest (blue).

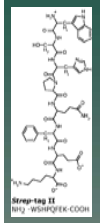


alkylguanine-DNA-alkyltransferase (AGT)

covalys

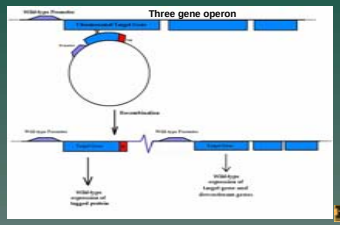
Tag proteins for Complex Isolation

Features of *Strep*-tag from IBA

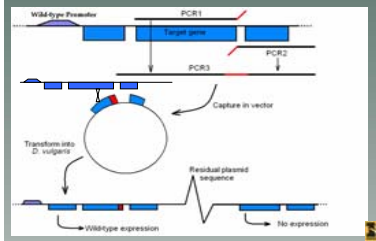


Removed with biotin analog desthiobiotin

Tagging procedure currently used for *D. vulgaris*



Tagging procedure for penultimate gene in operon



To date:

DVU number	Description	Strain Name
DVU0811	DnaK	JW907-0811
DVU2928	RpoB	JW907-2928
DVU2929	RpoC	JW907-2929
DVU1577	HslV	JW907-1577
DVU0503	Pnp	JW907-0503
DVU3184	Rub (rubredoxin)	JW905-3184
DVU1116	hypothetical*	JW907-1116
DVU1120	hypothetical	JW907-1120
DVU1810	hypothetical	JW907-1810
DVU1816	hypothetical	JW907-1816
DVU0846	ApsB	JW905-0846
DVU1568	Fln	JW907-1568
DVU1397	Bfr	JW913-1397
DVU2441	HspC	in progress
DVU3212	Nox	in progress
DVU2291	CooH	in progress
DVU3150	RpsA	in progress
DVU2776	DsrC	in progress

Quality Control on Tagged Protein Constructs

- Sequence PCR construct both orientations
- Single colony isolation of *D. vulgaris* transformants
- PCR testing of *D. vulgaris* transformants
- Southern analysis of *D. vulgaris* transformants
- Unique pattern to distinguish from parent
- Unique pattern to distinguish from plasmid

Tandem affinity tags for better purification: STF

STF: Strep-tag, TEV, and 3xFLAG

GGT CTC ... ATT CCA ACT

ACT GCT AGC ... GAG CTC

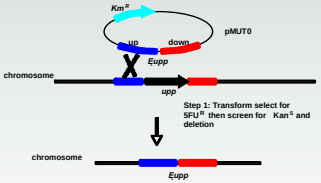
... TAG

Deletion Strains

Deletion construction with marker replacement

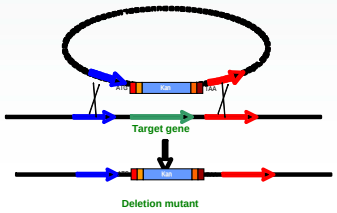
Mutagenic cassette

Creation of the Host *D. vulgaris* Strain for Unmarked Deletion Constructions



***D. vulgaris* JW710 was born!**

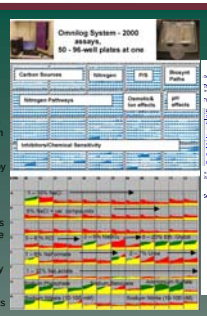
Transform *Desulfovibrio vulgaris*

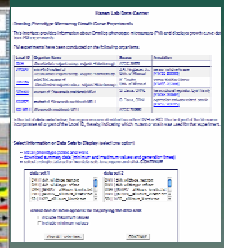


Current Progress in Deletion Construction

Deletion	Protein annotation	Mutant
Δfur	Global regulator, iron uptake	JW707
ΔperR	Global regulator, oxidation	JW708
Δzur	Global regulator, zinc uptake	JW709
Δupp	UPRTase	JW710
ΔnorM1	Na^+-dependent, multi-drug anti-porter pump	JW400
ΔDVU0164	cation diffusion facilitator	JW401
ΔnhaC-1	Na^+/H^+ antiporter	JW382
ΔnhaC-2	Na^+/H^+ antiporter	JW383
ΔnhaD	Na^+/H^+ antiporter	JW381
ΔmnhA	Na^+/H^+ antiporter (Ech hydrogenase, subunit EchA)	JW380
Δung	Uracil N-glycosylase	JW386

Phenotyping of Tagged Strains

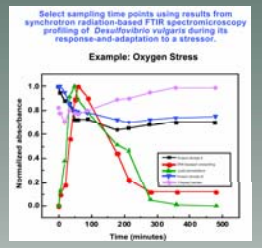




Phenotypic Microarray™ analysis is a recently developed analytical tool to determine the phenotype of an organism. This technique can be useful to understand the growth changes of an organism when changing medium, temperature, or adding a stressor, or when testing mutant strains . The plates, which are commercially available from Biolog™ (Hayward, CA), consist of an array of 20 plates. In our laboratory, we have developed the ability to use these plates under anaerobic conditions and are able to culture an obligate anaerobe, *Desulfovibrio vulgaris* successfully by inoculating the plate in an anaerobic chamber. The plates are then heat sealed in polyethylene bags containing an anaerobic sachet. It was found that preconditioning of the cells in specialized media was required for the different types of plates in order to get a valid phenotype. The plates have been successfully used to characterize the phenotype of the *D. vulgaris* Hildenborough ATCC 29579 and are currently being applied to mutant strains to provide rapid screening of mutant phenotypic changes, for rapid pathway analyses and modeling. See (Omnilog System - 2000 assays, 50 - 96-well plates at one time) for sample data sets and analyses.

Optimization of Growth Conditions and Scale-up

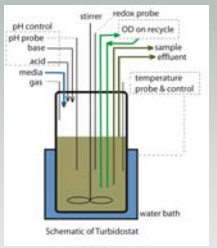
Culturing large quantities of biomass with the exact characteristics needed for the best characterization by the other subprojects will require a large number of growth curve studies and analysis at different time points using different techniques. In addition to classical techniques using measurements of direct microscopic cell counts, optical density, total protein concentrations, MPN, etc., we will automate and increase throughput by using the automated growth curves with the Omnilog phenotypic arrayer described above and using real-time analysis of cells under different conditions using synchrotron infrared spectromicroscopy. The Omnilog will allow us to screen more than 24 growth conditions on each 96-well plate. Since each well in each plate can be read every few minutes and 50 plates can be handled simultaneously, this will allow us to screen 1200 growth conditions in 80 h and more than 2400 every week. This will enable us quickly to find the optimal growth characteristics for each test condition for each tagged and wildtype strain of interest. The optimal conditions will then be scaled up in 1 liter batch cultures and then in 1 liter chemostats and finally in 3 liter or 7 liter full-scale chemostats. Though this technique works well and had been used by the VIMSS project for the past 2 years it does not provide critical information on the best time for sampling for desired proteins or cell changes to maximize recovery of the target proteins that. The synchrotron infrared spectromicroscopy provides real-time analyses that will characterize the physiological states of cells and to optimize harvesting times.



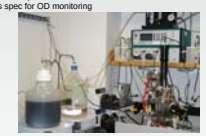
Large-Scale Biomass Production and Harvesting

1. Maintenance and archiving of *D. vulgaris* experimental strain (ATCC 29579) to minimize 'culture' drift from multiple transfers
2. Developed and defined growth conditions for stress studies using defined media (1000's of growth curves using automated systems)
3. QA/QC on all media components and culture conditions, e.g. pH, DO, temperature, containers, anaerobic chamber, etc.
4. Physiological and Morphological typing: PLFA, s-FTIR, AODC, Fluorescent Antibody (O&H),Omnilog (Phenotype Microarray), SEM, TEM, x-ray, chemotaxis, protein, optical density, lactate/acetate, sulfate, etc.
5. Developed and validated large scale production of biomass under sterile, anaerobic, reproducible conditions for simultaneous comparison of control and stress. Currently up to 2 L in triplicate under 2 conditions. Expanding to multiple 3 L no-metal, anaerobic chemostats.
6. Developed techniques for stressing cells that minimize other stress responses: e.g. log phase cells, sparge with N and air for comparison of air stress, special porous tubing to maximize DO saturation from air.
7. Developed sampling and processing techniques that minimize contamination, processing, shipping, and maximize sample quality and quantity for simultaneous sampling and processing for all subprojects.

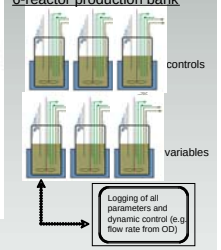
Biomass Production with Turbidostats



3-5 L capacity/unit
Non-metallic wetted parts
Ocean Optics spec for OD monitoring



6-reactor production bank



Summary

During the first 3 months, the MS has supplied the EM project with several sets of DvH on biofilms for analysis, three 5-liters of DvH biomass for water-soluble protein complex purification studies, and 120 l of DvH biomass for membrane protein complex purification. We started using tandem affinity tagging of proteins using three distinct tags in order to purify the protein of interest for detailed characterization. The first uses a "Strep-tag" that inserts a streptavidin binding peptide easy enrichment and we have currently constructed 16 such tags. However, in order to attain even higher protein enrichment, we are assessing the proven approach of a CTF (a.k.a. SPA) tag that includes a calmodulin binding protein (CBP), a protease (tobacco etch virus) and a 3 x FLAG site for monoclonal antibody binding versus an "STF" tag that replaces CBP with a streptavidin binding peptide. At issue is the hypothesis that since the latter is only 8 amino acids compared to 125 with CBP, it should be less likely to interfere with localization/orientation of the protein within the cell. All three approaches are currently being assessed with DsrC (DVU2776), a protein in the dissimilatory sulfite reductase pathway that is essential for cell growth via sulfate respiration. Once we have confidently determined the best approach, we intend to tag at least 60 selected proteins in the coming year. Twenty of these proteins will also be tagged with a peptide including a tetracysteine motif that allows in situ imaging. Since the chemistry upon which this tag is based is thiol chemistry, we must first establish that the sulfide generated by these bacteria does not irrevocably interfere. FtsZ (DVU2499), a cell division protein, is the first candidate for testing the efficacy of this procedure. Within the time scope of this project, we intend to differentially tag >300 of the gene products in *D. vulgaris*. This information is expected lead to a more thorough understanding of not only the proteins involved in metal-reduction but also their protein-protein interactions and characterization of the complete pathway(s) for these activities.

ACKNOWLEDGEMENTS

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